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(54) **Valproic acid and derivatives thereof as histone deacetylase inhibitors**

(57) The present invention relates to the use of valproic acid and derivatives thereof as an inhibitor of histone deacetylases. The invention also concerns the use of those compounds for the manufacture of a medicament for the treatment of diseases associated with histone hypoacetylation or in which a therapeutic effect can be achieved by induction of increased histone acetylation, such as tumors or leukemias, myelodysplasic syndrome or thyroid resistance syndrome. Also provided are methods for screening compounds having histone deacetylase activity using valproic acid/derivative thereof, and methods for identifying genes induced by valproic acid/derivatives thereof.

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Description

[0001] The present invention relates to the use of the drug valproic acid and derivatives thereof as inhibitor of enzymes having histone deacetylase activity. The invention also relates to the use of those compounds for the manufacture of a medicament for the treatment of diseases which are associated with hypoacetylation of histones or in which induction of hyperacetylation has a beneficial effect.

[0002] Local remodelling of chromatin is a key step in the transcriptional activation of genes. Dynamic changes in the nucleosomal packaging of DNA must occur to allow transcriptional proteins contact with the DNA template. One of the most important mechanisms contributing to chromatin remodelling is the posttranslational modification of histones by acetylation. Change in electrostatic attraction for DNA and steric hindrance introduced by the hydrophobic acetyl group leads to destabilisation of the interaction of histones with DNA. As a result, acetylation of histones disrupts nucleosomes and allows the DNA to become accessible to the transcriptional machinery. Removal of the acetyl groups allows the histones to bind more tightly to DNA and to adjacent nucleosomes and thus maintain a transcriptionally repressed chromatin structure. Acetylation is mediated by a series of enzymes with histone acetyltransferase (HAT) activity. Conversely, acetyl groups are removed by specific histone deacetylase (HDAC) enzymes. Disruption of these mechanisms gives rise to transcriptional misregulation and may lead to leukemic transformation.

[0003] Nuclear hormone receptors are ligand-dependent transcription factors that control development and homeostasis through both positive and negative control of gene expression. Defects in these regulatory processes underlie the causes of many diseases and play an important role in the development of cancer.

[0004] Several members of the nuclear receptor superfamily have been reported to interact with basal transcription factors, including TFIIB. However, numerous lines of evidence indicate that nuclear receptors must interact with additional factors to mediate both activation and repression of target genes. A number of cofactors that associate with the ligand binding domains of estrogen (ER), retinoic acid (RAR), thyroid hormone (T3R), retinoid X (RXR), and other nuclear receptors have recently been identified. Putative coactivator proteins include SRC-1 / NCoA-1, GRIP1 / TIF2 / NCoA-2, p/CIP / ACTR / AIB1, CBP and a variety of other factors (reviewed in Xu et al., 1999, Curr Opin Genet Dev **9**, 140-147). Interestingly, SRC proteins as well as CBP have been shown to harbor intrinsic histone acetyltransferase activity and to exist in a complex with the histone acetylase P/CAF.

[0005] Many nuclear receptors, including T3R, RAR and PPAR, can interact with the corepressors N-CoR and SMRT in the absence of ligand and thereby inhibit transcription. Furthermore, N-CoR has also been reported to interact with antagonist-occupied progesterone and estrogen receptors. N-CoR and SMRT have been shown to exist in large protein complexes, which also contain mSin3 proteins and histone deacetylases. Thus, the ligand-induced switch of nuclear receptors from repression to activation reflects the exchange of corepressor and coactivator complexes with antagonistic enzymatic activities.

[0006] The N-CoR corepressor complex not only mediates repression by nuclear receptors, but also interacts with additional transcription factors including Mad-1, BCL-6 and ETO. Many of these proteins play key roles in disorders of cell proliferation and differentiation. T3R for example was originally identified on the basis of its homology with the viral oncogene v-erbA, which in contrast to the wildtype receptor does not bind ligand and functions as a constitutive repressor of transcription. Furthermore, mutations in RARs have been associated with a number of human cancers, particularly acute promyelocytic leukemia (APL) and hepatocellular carcinoma. In APL patients RAR fusion proteins resulting from chromosomal translocations involve either the promyelocytic leukemia protein (PML) or the promyelocytic zinc finger protein (PLZF). Although both fusion proteins can interact with components of the corepressor complex, the addition of retinoic acid dismisses the corepressor complex from PML-RAR, whereas PLZF-RAR interacts constitutively. These findings provide an explanation why PML-RAR APL patients achieve complete remission following retinoic acid treatment whereas PLZF-RAR APL patients respond very poorly. The hypothesis that corepressor-mediated aberrant repression may be causal for pathogenesis in APL is supported by the finding that trichostatin A, which inhibits histone deacetylase (HDAC) function is capable of overcoming the differentiation block in cells containing the PLZF-RAR fusion protein. Furthermore, a PML-RAR patient who had experienced multiple relapses after treatment with retinoic acid has recently been treated with the HDAC inhibitor phenylbutyrate, resulting in complete remission of the leukemia (Warrell et al., 1998, J. Natl. Cancer Inst. **90**, 1621-1625).

[0007] Additional evidence that histone acetylation plays a role in cancer comes from studies on the AML1-ETO oncoprotein and on chromosomal rearrangements involving the MLL locus (Redner et al., 1999, Blood 94, 417-428).

[0008] WO 99/37150 discloses a transcription therapy for cancer comprising administering a retinoid substance and an inhibitor of histone deacetylase.

[0009] Several compounds are known to be HDAC inhibitors. Butyric acid, or butyrate, was the first HDAC inhibitor to be identified. In millimolar concentrations, butyrate is not specific for HDAC, it also inhibits phosphorylation and methylation of nucleoproteins as well as DNA methylation. Its analogue phenylbutyrate acts in a similar manner. More specific are trichostatin A (TSA) and trapoxin (TPX). TPX and TSA have emerged as potent inhibitors of histone deacetylases. TSA reversibly inhibits, whereas TPX irreversibly binds to and inactivates HDAC enzymes. Unlike butyrate,

nonspecific inhibition of other enzyme systems has not yet been reported for TSA or TPX. TSA and TPX, however, exhibit considerable toxicity and are therefore of limited therapeutic use.

[0010] It is an object of the present invention to provide substances which can be useful in the treatment of cancer.

[0011] It was surprisingly found that valproic acid (VPA; 2-n-propylpentanoic acid) is capable of inhibiting histone deacetylase.

[0012] Valproic acid is a known drug with multiple biological activities which depend on different molecular mechanisms of action.

- VPA is an antiepileptic drug.
- VPA is teratogenic. When used as antiepileptic drug during pregnancy VPA can induce birth defects (neural tube closure defects and other malformations) in a few percent of born children. In mice, VPA is teratogenic in the majority of mouse embryos when properly dosed.
- VPA activates a nuclear hormone receptor (PPARδ).Several additional transcription factors are also derepressed but some factors are not significantly derepressed (glucocorticoid receptor, PPARα).
- VPA is hepatotoxic, which may depend on poorly metabolized esters with coenzyme A.

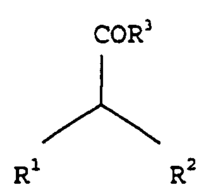
[0013] The use of VPA derivatives allowed to determine that the different activities are mediated by different molecular mechanisms of action. Teratogenicity and antiepileptic activity follow different modes of action because compounds could be isolated which are either preferentially teratogenic or preferentially antiepileptic (Nau et al., 1991, Pharmacol. Toxicol. 69, 310-321). Activation of PPARδ was found to be strictly correlated with teratogenicity (Lampen et al., 1999, Toxicol. Appl. Pharmacol. 160, 238-249) suggesting that, both, PPARδ activation and teratogenicity require the same molecular activity of VPA. Also, differentiation of F9 cells strictly correlated with PPARδ activation and teratogenicity as suggested by Lampen et al., 1999, and documented by the analysis of differentiation markers (Werling et al., manuscript in preparation). It is shown in the present application, that PPARδ activation is caused by the HDAC inhibitory activity of VPA and its derivatives. Furthermore it is shown that the established HDAC inhibitor TSA activates PPARδ and induces the same type of F9 cell differentiation as VPA. From these results we conclude that not only activation of PPARδ but also induction of F9 cell differentiation and teratogenicity of VPA or VPA derivatives are most likely caused by HDAC inhibition.

[0014] Antiepileptic and sedating activities follow different structure activity relationships and thus obviously depend on a primary VPA activity distinct from HDAC inhibition.

[0015] The mechanism of hepatotoxicity is poorly understood and it is unknown whether it is associated with formation of the VPA-CoA ester. The use according to the invention, e.g. HDAC inhibition, however, appears not to require CoA ester formation.

[0016] US patent No. 5,672,746 discloses the use of VPA and derivatives thereof for the treatment of neurodegenerative and neuroproliferative disorders.

[0017] One aspect of the present invention is therefore the use of VPA and derivatives thereof as an inhibitor of an enzyme having histone deacetylase activity. Derivatives of VPA are compounds of formula I



I

wherein R^1 and R^2 independently are a linear or branched, saturated or unsaturated aliphatic C_{1-25} hydrocarbon chain which chain optionally comprises one or several heteroatoms and which may be substituted, R^3 is hydroxyl, halogen, alkoxy or an optionally alkylated amino group.

[0018] Different R^1 and R^2 residues give rise to chiral compounds. Usually one of the stereoisomers has a stronger teratogenic effect than the other one (Nau et al., 1991, Pharmacol. Toxicol. 69, 310-321) and the more teratogenic isomer more efficiently activates PPARδ (Lampen et al, 1999). Therefore, this isomer can be expected to inhibit HDACs more strongly (this invention). Thus, different rules apply to the selection of substituents in the R^1 and R^2 position. The present invention encompasses the racemic mixtures of the respective compounds, the less active isomers, and in

particular the more active isomers.

[0019] The hydrocarbon chains R^1 and R^2 may comprise one or several heteroatoms (e.g. O, N, S) replacing carbon atoms in the hydrocarbon chain. This is due to the fact that structures very similar to that of carbon groups may be adopted by heteroatom groups when the heteroatoms have the same type of hybridization as a corresponding carbon group.

[0020] R^1 and R^2 may be substituted. Possible substituents include hydroxyl, amino, carboxylic and alkoxy groups as well as aryl and heterocyclic groups.

[0021] Preferably, R^1 and R^2 independently comprise 2 to 10 carbon atoms. It is also preferred that R^1 and R^2 independently are saturated or comprise one double bond or one triple bond. In particular, one of the side chains (R^1) may preferably contain sp^1 hybridized carbon atoms in position 2 and 3 or heteroatoms which generate a similar structure. This side chain should comprise 3 carbon or heteroatoms but longer chains may also generate HDAC-inhibiting molecules. Also inclusion of aromatic rings or heteroatoms in R^2 is considered to generate compounds with HDAC inhibitory activity because the catalytic site of the HDAC protein apparently accommodates a wide variety of binding molecules. With the novel observation that teratogenic VPA derivatives are HDAC inhibitors, also compounds which have previously been disregarded as suitable antiepileptic agents are considered as HDAC inhibitors under this invention. In particular, but not exclusively, compounds having a propinyl residue as R^1 and residues of 7 or more carbons as R^2, are considered (Lampen et al, 1999).

[0022] Preferably, the group "COR^3" is a carboxylic group. Also derivatization of the carboxylic group has to be considered for generating compounds with potential HDAC inhibitory activity. Such derivatives may be halides (e.g. chlorides), esters or amides. When R^3 is alkoxy, the alkoxy group comprises 1 to 25, preferably 1-10 carbon atoms. When R^3 is a mono- or dialkylated amino group, the alkyl substituents comprise 1 to 25, preferably 1-10 carbon atoms. An unsubstituted amino group, however, is preferred.

[0023] According to the present invention also pharmaceutically acceptable salts of a compound of formula I can be used.

[0024] The compounds which are most preferably used according to the present invention are VPA, S-4-yn VPA, EHXA (Ethyl hexanoic acid).

[0025] The compounds are useful for inhibiting mammalian (to cover cell lines for use in in vitro assays) and in particular human histone deacetylases HDAC 1-3 (class I) and HDAC 4-7 (class II).

[0026] The compounds may be used to induce the differentiation of cells such as undifferentiated tumour cells. Presumably, this reflects a general mechanism, as differentiation can be induced in F9 teratocarcinoma cells, MT 450 breast cancer cells, HT-29 colon carcinoma cells and several leukemia cell lines.

Another aspect of the present invention is the use of a compound of formula I for the manufacture of a medicament for the treatment of a disease which is associated with gene-specific hypoacetylation of histones. There are a number of diseases which are associated with aberrant repression of specific genes which correlates with a local level of histone acetylation below the regular level. Examples of such diseases are leukemia, epithelial teratocarcinoma, estrogen receptor-independent breast cancer, non-Hodgkin lymphoma, adrenocortical adenoma and thyroid resistance syndrome. In a preferred embodiment the compounds are used for the manufacture of a medicament for the treatment of acute promyelocytic leukemia (APL), acute myelocytic leukemia (AML) or MDS (myelodysplasia syndrome).

[0027] The compounds and salts thereof can be formulated as pharmaceutical compositions (e.g. powders, granules, tablets, pills, capsules, injections, solutions, foams, enemas and the like) comprising at least one such compound alone or in admixture with pharmaceutically acceptable carriers, excipients and/or diluents. The pharmaceutical compositions can be formulated in accordance with a conventional method. Specific dose levels for any particular patient will be employed depending upon a variety of factors including the activity of specific compounds employed, the age, body weight, general health, sex, diet, time of administration, route of administration, rate of excretion, drug combination, and the severity of the particular disease undergoing therapy. The active ingredient will preferably be administered in an appropriate amount, for example, selected from the range of about 10 mg/kg to 100 mg/kg body weight a day orally or intravenously. The dose levels are not specifically restricted as long as serum levels of 0.05 mM to 3 mM, preferably of about 0.4 mM to 1.2 mM are achieved.

[0028] Another aspect of the invention is a method for the identification of substances having histone deacetylase inhibitory activity which comprises providing a derivative of valproic acid, determining its histone deacetylase inhibitory activity, and selecting the substance if the substance has histone deacetylase inhibitory activity. Valproic acid can serve as a lead substance for the identification of other compounds exhibiting histone deacetylase inhibitory activity. Thereby compounds may be selected which show increased HDAC inhibitory activity at lower doses and serum levels and have decreased effects on the central nervous system such as sedating activity. Another parameter that may be optimised is the appearance of the hepatotoxic effect. Compounds may be selected which show a reduced liver toxicity. The derivatives may be provided by synthesising compounds which comprise additional and/or modified substituents. The HDAC inhibitory activity may be determined by a transcription repression assay, a Western Blot which detects acetylation of histone H3 and/or histone H4, or by an enzymatic assay.

[0029] The transcriptional assay for repressor activity exploits activation and derepression of a Gal4-dependent reporter gene. This assay can be performed either by transient transfection of mammalian cell lines (e.g. HeLa, 293T, CV-1) or with specifically constructed permanent cell lines. Transcription factors such as thyroid hormone receptor, PPARδ, retinoic acid receptor, N-CoR and AML/ETO repress transcription when they bind to a promoter containing UAS elements as fusion proteins with the heterologous DNA-binding domain of the yeast Gal4 protein. In the absence of the Gal4-fusion protein the reporter gene has a high basal transcriptional activity due to the presence of binding sites for other transcription factors in the thymidine kinase promoter. The Gal4 fusion proteins repress this activity by up to 140-fold. HDAC inhibitors induce relief of this repression which can be detected as an increase in reporter gene activity (e.g. by luciferase assay).

[0030] Histone deacetylase inhibitors induce the accumulation of N-terminally hyperacetylated histones H3 and H4. These acetylated histones can be detected by Western blot analysis of whole cell extracts or of histone preparations from histone deacetylase inhibitor-treated cells using antibodies specific for the acetylated N-terminal lysine residues of histones H3 and H4.

[0031] The enzymatic assay for HDAC activity records the release of ^{3}H-labeled acetic acid from hyperacetylated substrates. Sources of HDAC activity can be co-immunoprecipitates with antibodies directed against N-CoR (or other repressors known to recruit HDACs) or crude cell extracts containing histone deacetylases (e.g. HeLa, 293T, F 9). Substrates may be either chemically ^{3}H-acetylated peptides corresponding to the N-termini of histones H3 or H4 or histone proteins isolated from metabolically labelled cells which were treated with HDAC inhibitors. After extraction with ethyl acetate the release of ^{3}H-labeled acetic acid is detected by liquid scintillation counting.

[0032] Another aspect of the invention is the use of VPA or derivatives thereof to define genes which are induced by said compounds in cells such as primary human or rodent cells, leukemic cells, other cancer cells or tumor cell lines. The invention therefore provides a method for the identification of genes induced by VPA or a derivative thereof which comprises providing two populations of cells which are substantially identical, contacting one of the populations with VPA or a derivative thereof, and detecting genes or gene products which are expressed in the population which had been contacted with VPA or a derivative thereof at a level significantly higher than in the population which had not been contacted with VPA or a derivative thereof. Methods to define such genes that are induced by VPA include established technologies for screening large arrays of cDNAs, expressed sequence tags or so-called unigene collections. Also the use of subtractive hybridization techniques is suitable to define genes which are induced by VPA or derivatives thereof. The use of these methods to identify potential targets for drug development downstream of HDAC-inhibition is part of this invention. Considering the low general toxicity of VPA in the organism compared to other HDAC-inhibitors it is a specific aspect of this invention to use VPA or derivatives thereof for defining target genes which are selectively regulated by either VPA or other HDAC inhibitors like trichostatin A.

[0033] The present invention provides novel possibilities to treat various cancer diseases. Applicant found that VPA and derivatives thereof are potent HDAC inhibitors. The HDAC inhibitors known so far are either nonspecific like butyrate, or toxic like TSA and TPX. VPA has the advantage that it is already an approved drug for the treatment of epilepsy. Thus, a vast amount of data concerning side effects and pharmaceutical acceptability are available.

[0034] **Figure 1** describes the histone deacetylase inhibitor-like activation of PPARδ by VPA (example 1).

[0035] **Figure 2** shows that VPA activates several transcription factors in addition to PPARδ (example 2).

[0036] **Figure 3** shows VPA-induced accumulation of hyperacetylated histones H3 and H4 (example 3).

[0037] **Figure 4** shows the biochemical analysis of histone deacetylase activity in the absence or presence of VPA (example 4).

[0038] **Figure 5** shows indicators of VPA induced differentiation in HT-29 colonic carcinoma cells and F9-teratocarcinoma cells. The phenotypes of F9-teratocarcinoma cells differentiated by VPA or the histone deacetylase inhibitor trichostatin A appear identical (example 5).

[0039] The following examples further illustrate the invention.

Example 1

Activation of a PPARδ-glucocorticoid receptor hybrid protein by VPA

[0040] A reporter gene cell line for activation of the PPARδ ligand binding domain was constructed in CHO cells. A subclone of CHO cells was used which contained a transgenic reporter gene expressing a secreted form of the human placental alkaline phosphatase under control of the glucocorticoid receptor-dependent LTR-promoter of the mouse mammary tumor virus (Göttlicher et al. (1992) Proc. Natl. Acad. Sci. USA 89, pp. 4653-4657). A hybrid receptor comprising the amino-terminus of the glucocorticoid receptor fused to the ligand binding domain of PPARδ was expressed in these cells essentially as described for the expression of the corresponding hybrid of PPARα (Göttlicher et al., 1992, ibd.). The ligand binding domain of PPARδ was used starting at amino acid 138 as deduced from the sequence published by Amri et al. (J. Biol. Chem. 270 (1995) pp. 2367-2371). Activation of the PPARδ ligand binding domain in these

cells induces expression of the alkaline phosphatase reporter gene which is detectable by an enzymatic assay from the cell culture supernatant. Similar cells expressing the full length glucocorticoid receptor served as negative controls for specificity of receptor activation. For the experiment shown in figure 1 the PPARδ hyrid receptor expressing cells were seeded at 20 % confluency into 24-well culture dishes and treated for 40 h with the PPARδ ligand carbocyclic prostaglandin I_2 (PGI, 5 μM), VPA (1 or 2 mM), or the histone deacetylase inhibitors sodium butyrate (B, 0,2-5 mM) and trichostatin A (TSA, 300 nM). Reporter gene activity was monitored by an enzymatic assay (alkaline phosphatase). Values except for butyrate are means ± S.D. from triplicate determinations in 2 independent experiments which were normalized according to cPGI-induced activity (figure 1). The highly synergistic activation of the reporter gene by VPA together with the PPARδ ligand cPGI (P+V) which is similar to the synergistic activation by Trichostatin A together with cPGI (P+T), and the lack of synergism with trichostatin (T+V) or butyrate (not shown) indicate that VPA does not act like a bona fide ligand to PPARδ. VPA rather affects PPARδ activity by a mechanism which lies in the same sequence of events by which also the inhibitors of corepressor-associated histone deacetylases induce transcriptional activity of PPARδ.

Example 2

Activation of transcriptional repressors by VPA

[0041] The transcription factors thyroid hormone receptor (TR), peroxisome proliferator activated receptor δ (PPARδ), retinoic acid receptor (RAR), the corepressor N-CoR and the AML/ETO fusion protein repress transcription when they bind to a promoter containing UAS sites (Gal4 response element) as fusion proteins with the heterologous DNA binding domain of the yeast Gal4 protein. In the absence of the Gal4 fusion protein a luciferase reporter gene is transcribed at a high basal level due to the presence of binding sites for other transcription factors in the thymidine kinase (TK) promoter. Hela cells were transfected with a UAS TK luciferase reporter plasmid (Heinzel et al., 1997, Nature 387, pp 43-48) and expression plasmids for the indicated Gal4 fusion proteins using the calcium phosphate precipitate method. After 24 h the medium was changed and cells were incubated with histone deacetylase inhibitors for a further 24 h. Transcriptional repression is measured as luciferase activity relative to the baseline of cells transfected with an expression plasmid for the Gal4 DNA binding domain alone (Figure 2). The Gal4 fusion proteins repress this baseline activity by up to 140 fold. VPA at a concentration of 1 mM (close to the serum levels which are reached during therapeutic use) induces relief of this repression which is indicated as an increase in reporter gene activity. A relief of repression is also found after treatment with established histone deacetylase inhibitors (10 nM Trapoxin, 100 nM TSA) as well as after partial activation of TR and PPARδ by their respective ligands. A combination of ligand and HDAC inhibitors (including VPA) results in a synergistic effect, indicating that different molecular mechanisms are involved. Figure 2 shows that VPA affects the activity of several distinct transcription factors and cofactors. This finding suggests that VPA acts on a common factor in the regulation of gene expression such as corepressor-associated histone deacetylases rather than on individual transcription factors or receptors (e.g. as a ligand).

Example 3

Accumulation of hyperacetylated histones in VPA-treated cells

[0042] VPA and established histone deacetylase inhibitors like sodium butyrate (NaBu) or trichostatin A (TSA) induce the accumulation of hyperacetylated histones H3 and H4. These acetylated histones can be detected by Western blot analysis in cell extracts of appropriately treated cells. Figure 3 shows the results of such an analysis from a representative experiment. In this experiment both the time course of VPA-induced hyperacetylation (A) and the required VPA concentration (B) were determined.

(A) For the time course analysis F9 cells were seeded into 6-well culture dishes 30 h before the intended time point of analysis. Individual cultures were treated at the indicated time points before analysis by addition of 10-fold concentrated stock solutions in culture medium of VPA or trichostatin A. Whole cell extracts were prepared by rinsing the cell cultures twice in ice-cold phosphate buffered saline and lysis of cells in 250 μl of sample buffer for denaturing SDS gel electrophoresis. DNA of collected samples was sheared by sonication and samples were separated on a 15% denaturing polyacrylamide gel. Acetylated histones H3 and histone H4 were detected by Western blot analysis using commercially available antibodies (Upstate Biotechnology) specific for the acetylated forms of histones (Ac-H3, Cat-Nr.: 06-599; Ac-H4, Cat-Nr.: 06-598). Equal loading of the lanes was confirmed by staining a part of the polyacrylamide gel by Coomassie blue.

(B) For determination of the required VPA dose F9 cells were cultured in 6-well culture dishes for 8 h prior to

addition of VPA at the indicated concentrations. Whole cell extracts were prepared 16 h after treatment as described above. Analysis for acetylated histones H3 and H4 was performed as described in (A). VPA concentrations in the range of blood serum levels reached during therapeutic use of VPA as antiepileptic agent in humans induce hyperactylation of histones H3 and H4. At serum levels only slightly exceeding those intended for antiepileptic therapy VPA induces histone hyperacetylation as efficiently as sodium butyrate or trichostatin A used at concentrations which are expected to have a maximum effect. This experiment indicates that VPA or a metabolite formed in F9 cells inhibits histone deacetylase activity.

Example 4

VPA inhibits histone deacetylase activity *in vitro*

[0043] Immune precipitates from whole cell extracts using antibodies against the corepressor N-CoR or mSin3 contain histone deacetylase activity. This enzymatic activity is measured by incubating the immune precipitates with radioactively acetylated histone substrates from cells in which histones have been hyperacetylated in the presence of ^{3}H-acetate. The release of ^{3}H-acetate is detected as a measure of enzymatic activity by extraction with ethyl acetate and subsequent liquid scintillation counting (Figure 4). Addition of the histone deacetylase inhibitor trichostatin A (TSA, 10^{-7} M) to the reaction in vitro severely inhibits the enzymatic activity. VPA (0.2 mM, 1 mM, 5 mM) and the related compounds ethyl hexanoic acid (EHXA, 0.008 mM, 0.04 mM, 0.2 mM, 1 mM, 5 mM), R-4-yn VPA (0.2 mM, 1 mM, 5 mM) and S-4-yn VPA (0.2 mM, 1 mM, 5 mM) were tested for HDAC inhibitory activity. The assays were performed with N-CoR immunoprecipitates from 293T cells in duplicate. Immunoprecipitates were pretreated with HDAC inhibitors for 15 min prior to the addition of substrate and subsequent incubation for 2.5 h at 37°C (untreated enzyme activity 2,205 cpm = 100%). Precipitates of a preimmune serum served as a negative control. EC_{50} values are 0.6 mM for VPA, 0.2 mM for EHXA and 0.3 mM for S-4-yn VPA, whereas the stereoisomer R-4-yn VPA is inactive. These data show that VPA by itself rather than a cellular metabolite inhibits histone deacetylase activity.

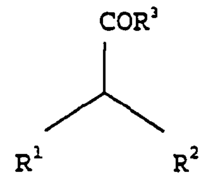
Example 5

Induction of cell differentiation in F9 teratocarcinoma, MT-450 breast cancer cells and HT-29 colonic cancer cells

[0044] Histone deacetylase inhibitors and VPA in particular induce differentiation of dedifferentiated tumorigenic cells. Cell differentiation is associated with a decrease in cell proliferation, morphological alterations and the appearance of expression of markers of the differentiated phenotype. The reduction of proliferation rate was shown in three examples, e.g. F9 teratocarcinoma, estrogen independent MT-450 breast cancer and HT-29 colonic carcinoma cells. F9 and HT-29 cells were cultured for 36 h in the absence or the presence of 1 mM VPA in 96-well culture dishes. 37 kBq of ^{3}H-thymidine were added for additional 12 h of culture. MT-450 cells were cultured for 72 h prior to a 1 h ^{3}H-thymidine labelling period. Incorporation of ^{3}H-thymidine into DNA was determined by automatic cell harvesting and liquid scintillation counting. VPA pretreatment reduced the rate of thymidine incorporation by 48 ± 5%, 63 ± 8%, and 52 ± 8% in F9, MT-450, and HT-29 cells, respectively. The dose-response for the reduction of thymidine incorporation into HT-29 cells (Figure 5A) was determined by the same experimental procedure. The induction of a cell differentiation marker was shown in F9 teratocarcinoma cells (Figure 5B).
F9 teratocarcinoma cells were treated for 48 h with VPA (1 mM), sodium butyrate (B, 1 mM) and trichostatin A (TSA, 30 nM). Differentiation was followed by morphological criteria, a reduced rate in the increase of cell number (data not shown), the drop of ^{3}H-thymidine incorporation by 48% during a 12 h pulse labeling period (see above) and the appearance of nuclear AP-2 protein (Figure 5B) as a specific marker of histone deacetylase inhibitor-induced differentiation of F9 cells. Nuclear AP-2 protein was detected in nuclear extract which had been prepared by mild detergent lysis (25 mM Tris, pH 7.5; 1 mM EDTA, 0.05% NP40) of treated or non-treated F9 cells, recovery of nuclei by centrifugation (3000 x g, 5 min) and lysis of nuclei in sample buffer for denaturing SDS gel electrophoresis. Nuclear extracts were separated on a 9 % SDS polyacrylamide gel. AP-2 protein was detected by Western blot analysis using a rabbit polyclonal antibody (Santa Cruz, Cat.-No.: SC-184) at a dilution of 1/1000 in Tris buffered saline containing 3% nonfat dry milk and 0.05% Tween 20. Both VPA and trichostatin A induce nuclear AP-2 protein whereas the activity of butyrate at the chosen concentration is weak. Since appearance of AP-2 is a delayed effect which is only detectable after 36 to 40 h of VPA treatment the weak activity of butyrate may be caused by efficient metabolism of the compound. Nevertheless, VPA induces differentiation of the epithelial F9 cell line in a way indistinguishable from differentiation by other histone deacetylase inhibitors.

Claims

1. The use of a compound of formula I



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wherein R^1 and R^2 independently are a linear or branched, saturated or unsaturated, aliphatic C_{1-25} hydrocarbon chain which optionally comprises one or several heteroatoms and which may be substituted, R^3 is hydroxyl, halogen, alkoxy or an optionally alkylated amino group, or of pharmaceutically acceptable salts thereof as an inhibitor of an enzyme having histone deacetylase activity.

2. A use according to claim 1, wherein R^1 and R^2 independently are a linear or branched C_{2-10} hydrocarbon chain which chain optionally comprises one double or triple bond.

3. A use according to claim 1 or 2, wherein the compound is selected from the group consisting of VPA, S-4-yn VPA and EHXA (Ethyl hexanoic acid).

4. A use according to anyone of claims 1 to 3, wherein the compound is valproic acid.

5. A use according to anyone of claims 1 to 4, wherein the enzyme having histone deacetylase activity is a mammalian, preferably a human histone deacetylase.

6. A use according to claim 5, wherein the human histone deacetylase is selected from the group consisting of HDACs 1-7.

7. A use according to anyone of claims 1 to 6, wherein the compound is used for the induction of differentiation of cells.

8. The use of a compound as defined in claims 1 to 4 for the manufacture of a medicament for the treatment of a disease which is associated with local gene-specific hypoacetylation of histones.

9. A use according to claim 8, wherein the disease is selected from the group consisting of leukemia, epithelial teratocarcinoma, estrogen receptor-independent breast cancer, non-Hodgkin lymphoma, adrenocortical adenoma, colon cancer, and thyroid resistance syndrome.

10. A use according to claim 9, wherein the disease is selected from the group consisting of acute promyelocytic leukemia (APL), acute myelocytic leukemia (AML), and myelodysplasia syndrome (MDS).

11. A method for the identification of substances having histone deacetylase inhibitory activity which comprises

    providing a derivative of valproic acid;

    determining the histone deacetylase inhibitory activity of the derivative;

    selecting the substance if the substance has histone deacetylase inhibitory activity.

12. A method according to claim 11, wherein the method comprises the steps of

    determining the sedating effect of the substance and

    selecting the substance if the substance has histone deacetylase inhibitory activity and a lower sedating effect than valproic acid.

13. A method according to claim 11 or 12, wherein the histone deacetylase inhibitory activity is determined by

a transcription repression assay,

a Western Blot detecting acetylation of histone H3 or histone H4,

or by enzymatic deacetylase assay.

14. A method for the identification of genes induced by valproic acid or a derivative thereof which comprises

providing two populations of cells which are substantially identical;

contacting one of the populations with VPA or a derivative thereof;

detecting genes or gene products which are expressed in the population which had been contacted with VPA or a derivative thereof at a level significantly higher than in the population which had not been contacted with VPA or a derivative thereof.

15. A method according to claim 14, wherein substractive hybridization or screening of arrays of cDNA samples, expressed sequence tags or unigene collections is employed.

Figure 1

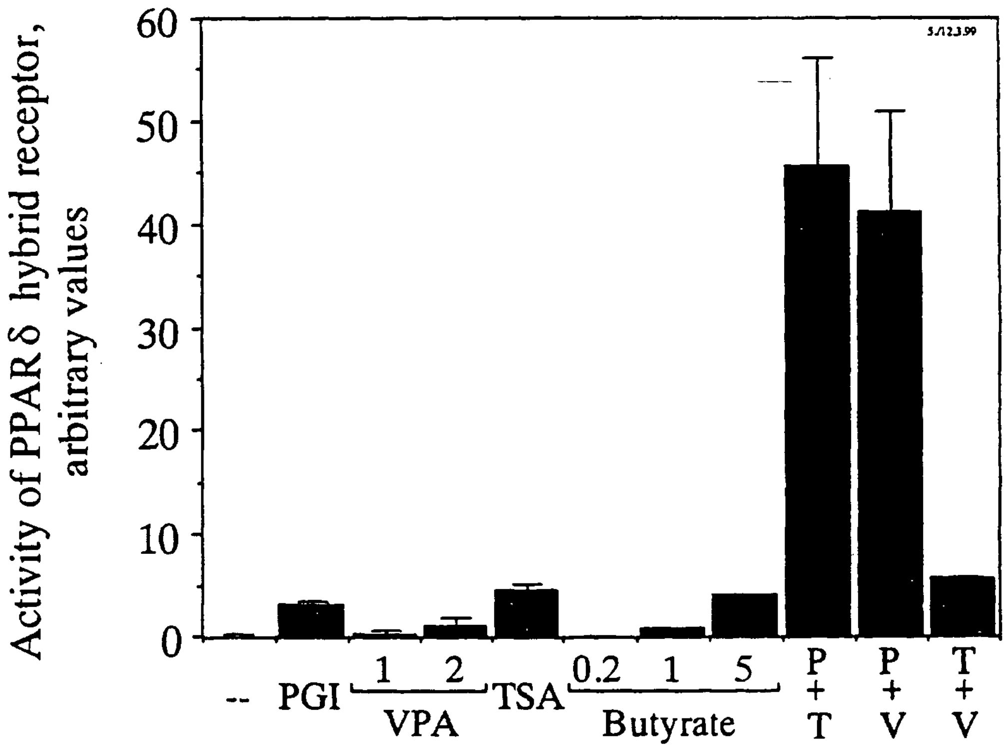

Activity of PPAR δ hybrid receptor, arbitrary values
60
50
40
30
20
10
0
--
PGI
1
2
VPA
TSA
0.2
1
5
Butyrate
P + T
P + V
T + V

Figure 2

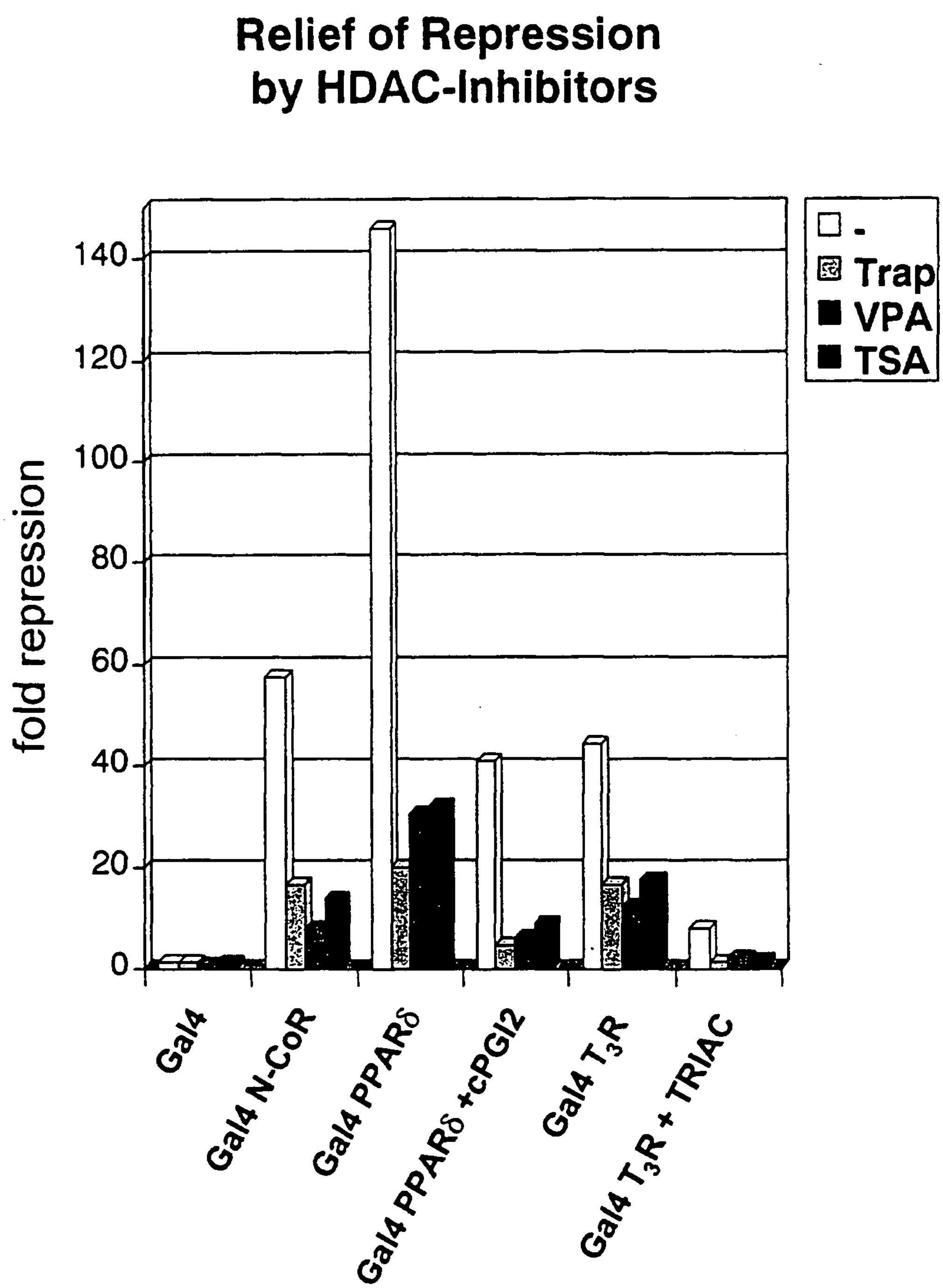

Relief of Repression
by HDAC-Inhibitors
fold repression
0
20
40
60
80
100
120
140
-
Trap
VPA
TSA
Gal4
Gal4 N-CoR
Gal4 PPARδ
Gal4 PPARδ +cPGI2
Gal4 T3R
Gal4 T3R + TRIAC

Figure 3

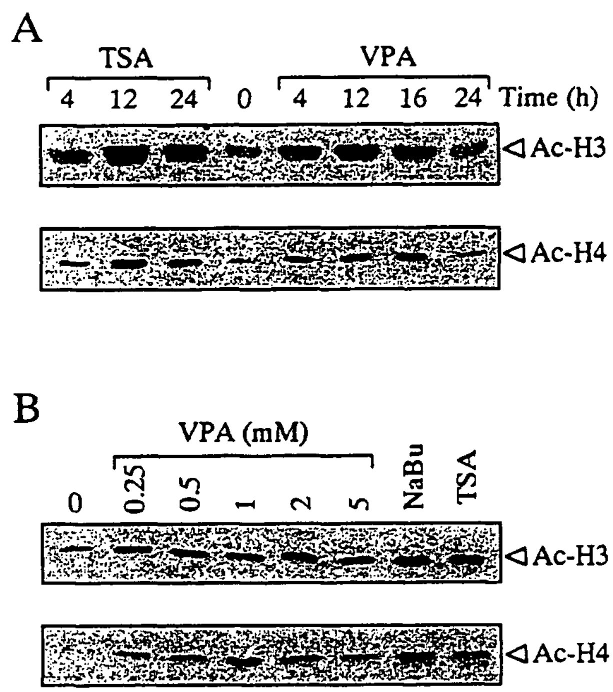

A
TSA
VPA
4 12 24 0 4 12 16 24 Time (h)
Ac-H3
Ac-H4
B
VPA (mM)
0 0.25 0.5 1 2 5 NaBu TSA
Ac-H3
Ac-H4

Figure 4

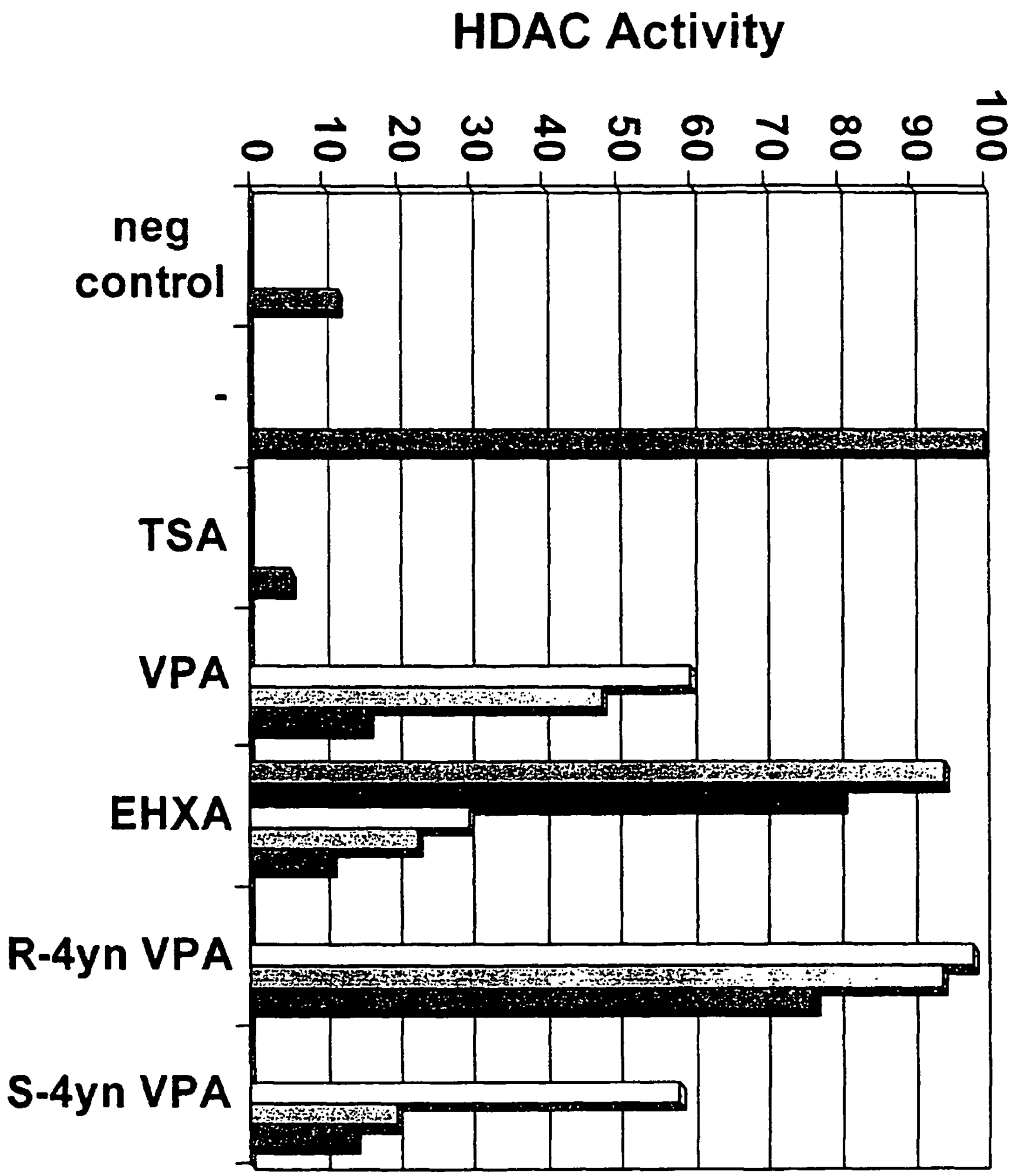

HDAC Activity
0
10
20
30
40
50
60
70
80
90
100
neg control
-
TSA
VPA
EHXA
R-4yn VPA
S-4yn VPA

Figure 5A

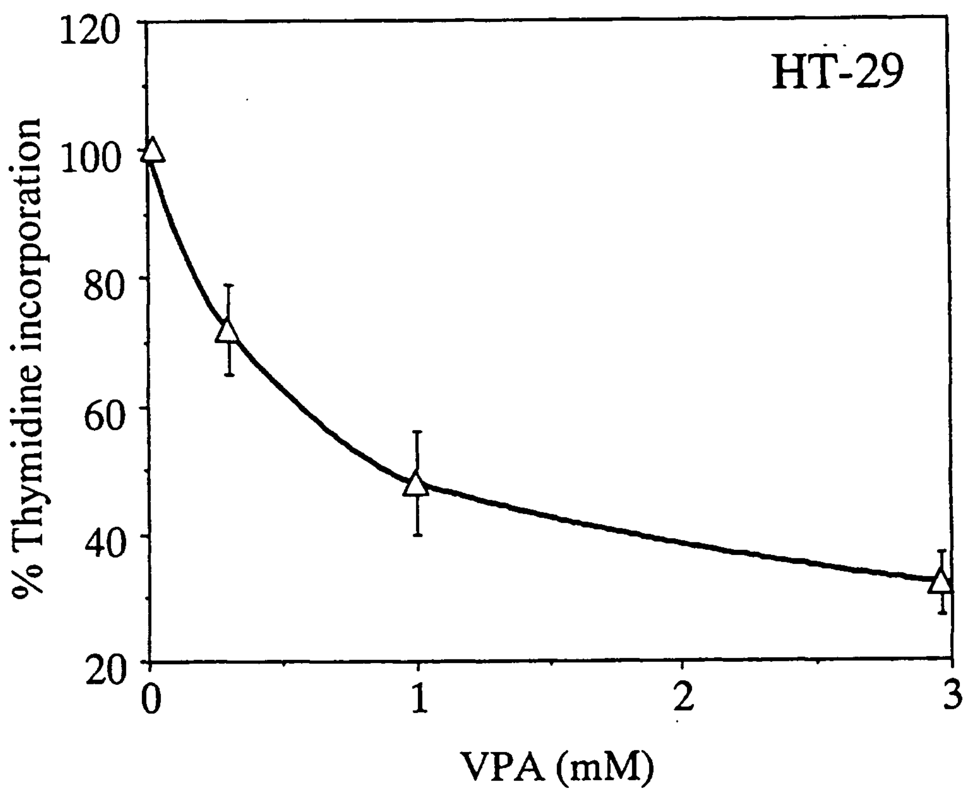
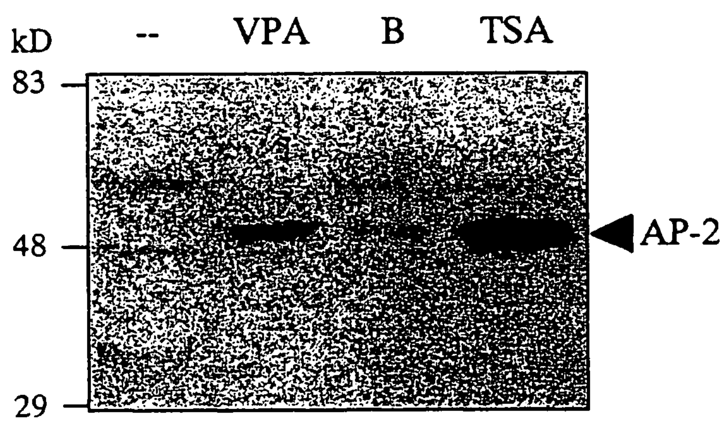


Figure 5B



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EUROPEAN SEARCH REPORT

Application Number
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Y	---	13	
Y	WO 99 23885 A (THE SALK INSTITUTE FOR BIOLOGICAL STUDIES) 20 May 1999 (1999-05-20) * the whole document,especially page 9 lines 14-28, page 10, pages 30-34 and claims 28-48 *	1,2,13	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
A	---	3-10	A61K C12Q
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~~The present search report has been drawn up for all claims~~

Place of search	Date of completion of the search	Examiner
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Application Number
EP 00 11 4088

CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

☐ Only part of the claims have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid, namely claim(s):

☐ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

see sheet B

☐ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.

☐ As all searchable claims could be searched without effort justifying an additional fee, the Search Division did not invite payment of any additional fee.

☐ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid, namely claims:

☒ None of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims, namely claims:

1-13 searched incompletely

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EUROPEAN SEARCH REPORT

Application Number
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Y	US 5 858 365 A (D.V. FALLER) 12 January 1999 (1999-01-12) * column 11 - column 15 *	1,2	
A	---	3,4,9,10	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
A,D	WO 99 37150 A (SLOAN KETTERING INSTIZUTE FOR CANCER RESEARCH) 29 July 1999 (1999-07-29) * the whole document * --- -/--	1-10	

~~The present search report has been drawn up for all claims~~

Place of search	Date of completion of the search	Examiner
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LACK OF UNITY OF INVENTION SHEET B

Application Number EP 00 11 4088

The Search Division considers that the present European patent application does not comply with the requirements of unity of invention and relates to several inventions or groups of inventions, namely:

1. Claims: 1-13

   Use of a compound of claim 3 as a histone deacetylase (HDA) inhibitor to treat diseases of claims 9 and 10, and methods for the identification of substances having HDA inhibitory activity using valproic acid.

2. Claims: 14-15

   Method of identifying genes induced by valproic acid using two populations of cells and measuring gene expression therein, as far as not already covered by previous subject.

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EUROPEAN SEARCH REPORT

Application Number
EP 00 11 4088

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Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
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~~The present search report has been drawn up for all claims~~

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